

Spectroscopic Evaluation of Freeze Damage in Pear Trees Using Fourth-Derivative Visible Spectroscopy of Bark Sections

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Methods previously used to describe plant germplasm by fourth-derivative visible spectroscopy of intact leaf lamina were modified to analyze in situ biochemical changes in bark tissues after freezing. Two bark characteristics were examined: photosynthetic apparatus and the process of tissue oxidation that follows freeze damage. Time sequences (or kinetics) of oxidation processes and the disorganization of light-harvesting systems are described. Changes in the spectral envelope of chlorophyll-protein complexes Cb 660 and Cb 670 were observed.

Room-temperature visible-range absorption spectroscopy of intact leaf lamina provides valuable information for genetic, germplasm, structural, and ecological studies (1-5). Fourth-derivative analysis of absorption spectra yields abundant information that can be used in these areas of research (2,6). In this article, the use of this technique to examine bark tissue will be discussed. One advantage of using absorption spectroscopy of intact bark tissues is that it is possible to rapidly determine if data obtained from other species and from different methods apply to the bark from the species of interest. This is particularly useful because much of the recent data on bark tissues is published in less accessible journals, such as some of those listed as references.

Bark tissue is used for two purposes at the National Clonal Germplasm Repository (NCGR) in Corvallis, Oregon: electrophoretic identification of germplasm (7) and cold hardness evaluations through differential thermal analysis (8). Although it is known that phenol oxidase inhibitors must be used to prepare good samples for electrophoretic analysis (7) and that accelerated phenol oxidase activity follows freezing (8,9), little is known about effects of phenol oxidase on the bark tissues after freezing.

Bark is composed of many elements, including water and mineral and metabolite conducting systems as well as photosynthetically active tissues (10). The year-round availability of stem tissues that express genetic characteristics of most genomes (nuclear, mitochondrial, and chloroplastic) is very important in isozyme characterization (7), because total isozyme expression depends on the presence of all these tissues (11).

EXPERIMENTAL

The instrument used was a model 260 spectrophotometer (Shimadzu Scientific Instruments, Columbia, Maryland) with an integrating sphere attachment, which has been previously described (2). The opening in the blackened copper mask in the holding device was changed from two circular holes (2) to a slot that was 29 mm long and 5 mm wide. The outer surface of a bark sample was placed facing the incident beam. The principal program used for analysis was based on the use of accumulated transmission spectra from multiple samples (6). Spectra (generated with a 5-nm slit width) from six different samples — where each spectrum was accumulated for about 7 min — were combined, noise was smoothed (12-16) using a running average

width of 4 nm (2), and fourth-derivative analysis was performed (6). Treatments included freezing and/or buffer addition and corresponding controls. *Pyrus* (pear) materials from two clones — CPYR 1274 and a *P. calleryana* open-pollinated-cross, PC-OB (7) — were obtained from the Corvallis NCGR collections in April, May, and June of 1987. Twig sections were collected in the evening and stored for use the following day. Twig sections, which were approximately 20 cm long, were stored in darkness at about 4 °C in plastic bags containing wet paper towels until required. After the twig sections were stored, all manipulations were carried out under low-light conditions (1-6 μ Einsteins/m²/s). Spectra were taken of the samples within 5 min of preparation and samples were then frozen immediately. One hour after freezing, the samples were thawed by immersion in distilled water. Spectra were then taken as soon as samples were thawed and at each hour for the next three hours. Samples were washed with distilled water before and after each spectrum was taken. After thawing, samples were stored between analyses under moist conditions at about 23 °C under less than 1 μ Einstein/m²/s of light. Buffer treatment samples were moistened with buffer after each water wash. The buffer was a modified extraction buffer (7), from which solid polyvinylpyrrolidone, mixed bed resin, and the toxic compound phenyl methyl sulfonyl fluoride had been omitted. The remaining buffer components (50 mM Tris-HCl, 1 mM sodium EDTA, 3 mM dithioerythritol, 5 mM ascorbic acid, 3 mM sodium metabisulfite, 6 mM diethyldithiocarbamate, and 8 mM glutathione) were titrated to pH 7.6. For at least 1 h, freezing treatments of -40 °C or colder

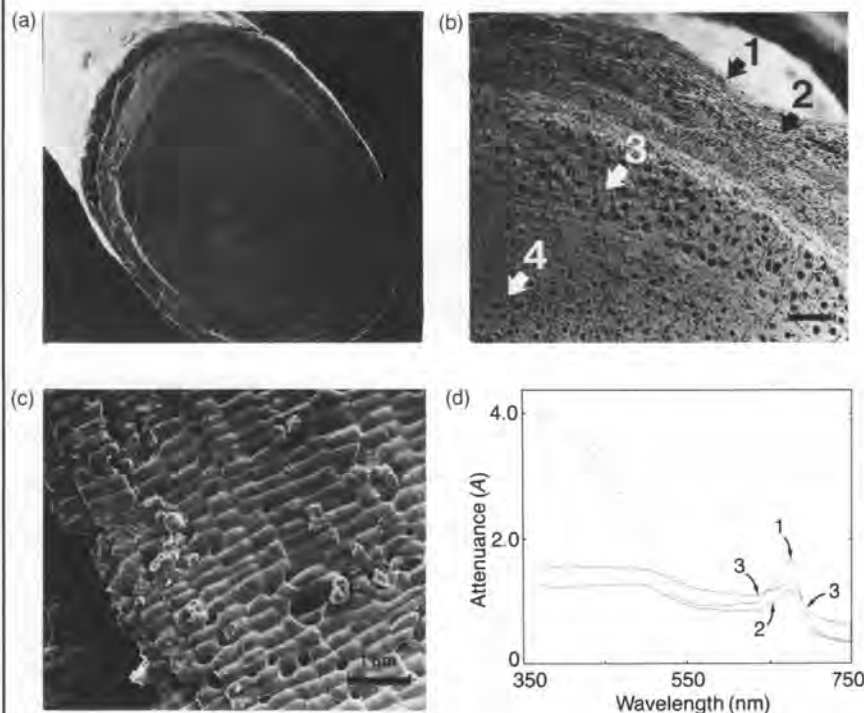


Figure 1. Plant material. (a) Scanning electron micrograph (14 \times) of a twig of clone PC-OB. (b) Scanning electron micrograph (100 \times) of a cross section of the twig. The numbered items are: 1 = phellem or peridermal layer; 2 = chlorophyll-containing layer used for experiments; 3 and 4 = layers of xylem tissue (wood) from previous years of growth. (c) Lateral view of tissue after phellem has been removed showing the layers of chlorophyll-containing cells (150 \times). (d) Spectra of phellem layer removed from material used: 1 = spectrum before scraping to remove chlorophyll-containing tissue; 2 = spectrum after partial removal of chlorophyll-containing tissue; 3 = spectrum after further removal of chlorophyll-containing tissue. Spectrum 3 has a higher attenuation at 750 nm, because during removal of last chlorophyll, the phellem tissue becomes very fragile and has to be placed in a spectroscopic sample holder where parts of several fragments are superimposed. For this reason, spectrum 3 should be considered approximately 1.75 \times and the amount of chlorophyll retained per unit area of phellem should be considered diminished by the same factor.

were applied to overcome supercooling of non-cold-adapted *Pyrus* tissue (8,9). Chlorophylls were determined by a standard method (17). Data were analyzed using nonparametric statistical methods (3,18).

RESULTS AND DISCUSSION

Stem photosynthetic tissues are covered by a peridermal layer of dead cells (19) or phellem (10). Figures 1a–1c show scanning electron micrographs of the tissues involved. At low magnification, Figure 1a shows a twig of the type from which the bark was obtained. Figure 1b shows a cross section of the twig where the external peridermal layer is immediately above a chlorophyll-rich layer, which is in turn above the phloem, the cambium layer, and the new xylem, all of which cover the xylem (or wood) produced in previous years. Figure 1c shows the layers of chlorophyll-rich cells. The peridermal layer is closely associated with chlorophyll-rich cells and only rigorous scraping can remove the character-

istic chlorophyll spectra from this layer (Figure 1d).

To prepare the sample, a piece of stem was cut a little longer than required for the sample holder (~ 35 mm). On this stem section, two incisions approximately 7–14 mm apart were made parallel to the vertical axis of the stem to yield, after removal of the stem, a section of bark that would be slightly wider than the aperture of the sample holder. Prior to removal of the bark segment, the peridermal layer was removed using fingernails. Then, also using fingernails, the bark carefully was peeled from the stem. This is done best in spring after the "sap rises" and the cambium (Figure 1b) becomes active. If the trees are well watered, it can also be accomplished in early summer.

When frozen pear bark thaws and the phellem is removed, strong phenol oxidase activity (or browning) is initiated (9). Figure 2

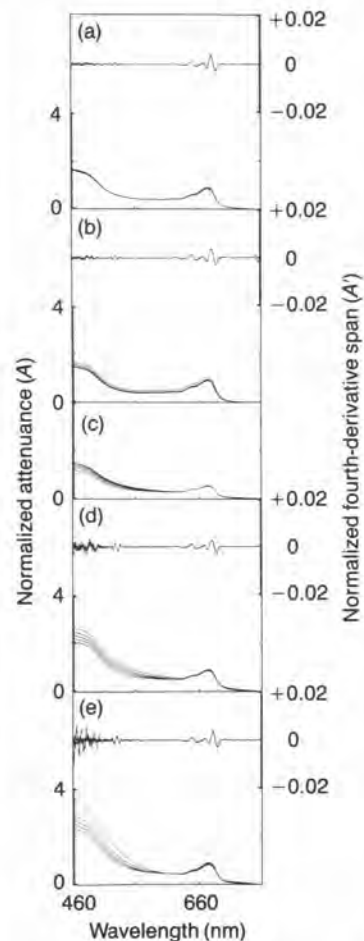


Figure 2. Time courses of oxidation of PC-OB bark tissue. The more abruptly changing curve, which starts in the upper quarter of the vertical axis on the right side, is the fourth-derivative trace. The less abruptly changing curve, which starts at the base of the vertical axis on the right side, is the attenuation (\sim absorbance) trace. Each spectrum is normalized to one attenuation unit at maximum red-chlorophyll absorption (~ 680 nm). Bark samples with their peridermal (or phellem) layer removed were abraded with a razor blade from about 1.1–1.5 A before treatment to about 0.8 A in order to permit analysis of oxidation effects at shorter wavelengths where absorption became much higher. One piece of bark tissue was used at a time, and replicate experiments were used to confirm effects (not shown). Except for spectrum (c), 12 accumulations were used for each trace to obtain a satisfactory fourth-derivative trace, and the interval between traces was 34 min. In spectrum (c), there was no fourth-derivative trace, and the interval between traces was ~ 4 min. Treatments for spectra include: (a) none, (b) buffer treated, (c) frozen short term, (d) frozen long term, and (e) frozen plus buffer.

Table 1. Chlorophyll content (\pm standard error of mean) of pear leaves and bark tissue. Statistical differences between bark and leaves of the same clone are indicated by: * ($P > 0.90$); † ($P > 0.95$); ‡ ($P > 0.99$). Data have been rounded after statistical calculations. Chlorophyll concentrations are expressed in mg/dm^2 .

Tissue	Clone	Chlorophyll		a/b ratio
		a	b	
Bark	CPYR 1274	0.73(± 0.12)†	0.30(± 0.05)†	2.4(± 0.0)‡
	PC-OB	0.71(± 0.16)‡	0.32(± 0.07)*	2.3(± 0.1)‡
Leaf	CPYR 1274	5.73(± 1.31)	1.60(± 0.35)	3.6(± 0.4)
	PC-OB	2.75(± 0.55)	0.72(± 0.13)	3.8(± 0.2)

shows the changes in spectral properties associated with this process. Three things are readily apparent: strong phenol oxidase activity occurs only after freezing (Figures 2c–2e); buffer addition enhances browning but only if freezing has occurred (Figures 2d and 2e); and the browning reactions do not significantly interfere with the spectra of chlorophyll–protein complexes (Figure 2e). It is evident that browning reactions are light activated or light enhanced. Bark tissue masked by the holder turned brown slowly, while tissue that was exposed to the spectroscopic light source (less than $1 \mu\text{Einstein}$ at 600 and 750 nm) browned rapidly during the first analysis after freezing (not illustrated). The addition of buffer that contained 5 mM ascorbate, which is an oxidase substrate, changed the spectra obtained and displaced the isosbestic point to shorter wavelengths (Figure 2e).

Statistical analysis (Table 1) indicates that bark and leaf chlorophyll compositions differ significantly. The difference between chlorophyll a and chlorophyll b ratios of bark and leaves is consistent with the data from *Syringa vulgaris* (20), but in *Pyrus*, bark chlorophyll concentrations are much less than those in *S. vulgaris*. Some of this difference may be attributable to the loss of chlorophyll when the peridermal layer is removed (Figure 1d). As reported previously for *Pinus sylvestris* (21), the chlorophyll content of *Pyrus* bark twigs in the illuminated and shaded portions of the tree canopy was not statistically different (Table 1). Spectral comparisons between leaf and bark chlorophyll–protein complexes were deferred because it was first necessary to investigate spectral stability of the bark preparations. It has been reported that chlorophyll–protein complexes of bark are less subject to diurnal dynamics than

complexes of leaves (22,23). Little is known about the function of bark chloroplasts (19), but these chloroplasts can fix carbon for processes that differ from those found in the leaves (24). A correlation between bark chlorophyll content and cold hardiness has been shown in *Malus* (apple), and chlorophyll content was also related to ascorbic acid levels in apple bark (25). Ascorbate metabolism is known to increase during cold acclimatization in other tree species (26). *Pyrus* and *Malus* are closely related members of the Poimodea family (27), and ascorbic acid is used to suppress phenol oxidase damage in protein extraction of bark of both species (7,28). Whether the amount of ascorbate that is present naturally — 1.6–3.0 mM, about equal in weight to that of chlorophyll in the related genus *Crataegus* (29) — is sufficient for this purpose is not known. The buffer used, however, contains 5 mM ascorbate, which presumably is responsible for the change in oxidation spectra (Figure 2e) and alters the effects of freezing on spectra of chlorophyll–protein structures (Figure 3).

Figure 3 illustrates the effects and interactions of the buffer and freezing treatments on the stability of the bark chlorophyll–protein spectra. The most apparent effect is a change in the envelope containing Cb 660 and Cb 670 (30–33). This area is shaded for better illustration. To define these differences, we use the term “spectral envelope coherence,” where a spectral envelope is “more coherent” when the component spectral bands are less apparent and “less coherent” when the component bands show more resolution. It is presumed that as the spectral envelope becomes more coherent, the interaction between the chromophores in the envelope increases. Because photosynthetic light-harvesting mechanisms require interaction between chromophores (34), this concept is consistent with the literature and the effects of freezing presented here (Figure 3b). To quantify the change in coherence of the Cb 660–Cb 670 spectral envelope in a complex biological system, nonparametric ranking statistical procedures — such as those used previously for *in vivo* descriptions of plant pigments (3) — were applied (18). Nonparametric linear regression using the rank of apparent envelope coherence was used. In this envelope for each experiment, the relative lack of fine structure in the bands was evaluated. Within each experiment, the envelope with the least apparent fine structure was given a rating of one and the one showing the most was given a rating of five. The replications of 2–4 spectral series for each treatment (data were taken from 12 to 24 bark segments) were pooled to produce the regression data for a plot of time (x axis) against the rank of envelope coherence (y axis). The resulting equations (rounded to three significant figures) were: no freezing, no buffer (Figure 3a) where $y = 2.93 + 0.25x$ and the correlation

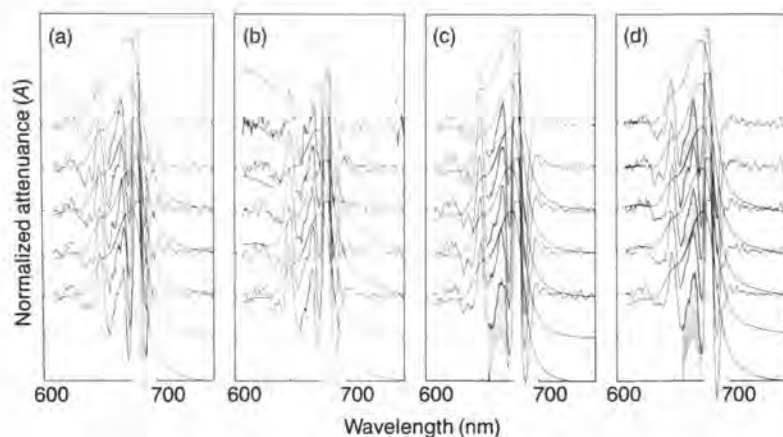


Figure 3. Effects of freezing and buffer treatment on spectra of PC-OB bark tissue. Spectra are normalized and represent a time series taken at times separated by 1 h. The upper spectrum is the last and the lowest is the first. Each portion of the figure represents the accumulated spectra of six bark samples; the same six bark samples were used for spectra in the same time series. The program used follows the standard method (6). Treatments include: (a) none, (b) frozen, (c) buffer treated, (d) buffer treated and frozen.

coefficient was 0.25; freezing, no buffer (Figure 3b) where $y = 0.15 + 0.97x$ and the correlation coefficient was 0.99; no freezing, plus buffer (Figure 3c) where $y = 1.80 + 0.40x$ and the correlation coefficient was 0.40; and freezing, plus buffer (Figure 3d) where $y = 1.5 + 0.50x$ and the correlation coefficient was 0.50. These data are consistent with the buffer producing a protective effect on the coherence of the spectral envelope containing Cb 660 and Cb 670, and thus the buffer presumably protects the integrity of the complex containing these chlorophyll-protein complexes. Minor effects of phenol oxidase activity can be observed, as indicated by the shading at shorter wavelengths in Figure 3b and to a lesser extent in Figure 3d. The lesser effect shown in Figure 3d is attributed to the displacement of the oxidation chromophores to shorter wavelengths by buffer addition (Figure 2e).

CONCLUSIONS

It is possible to follow enzymatic activities and changes in chlorophyll-protein structure in intact tissues of bark with spectrophotometric methods. The results obtained in these experiments demonstrate the induction of phenol oxidase activities following freezing and protection of protein-chlorophyll complexes by an appropriate buffer. In addition, the demonstration of chlorophyll-protein complexes in this tissue explains why *Pyrus* stem tissue has a large complement of isozymic forms.

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NOTE IN PROOF

It came to the attention of the authors that spectra of extracted leaf phenolics, induced by cold treatment, are soon to be published: L. Chalker-Scott, L.H. Fuchigami, and R.M. Harber, *HortScience* (1987), in press.

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